IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Stavrianopoulos et al.
Serial No. 08/486,070
Filed: June 7, 1995
Title: COMPOSITION EMPLOYING CHEMICLLY LABELED OLIGONUCLEOTIDE OR POLYNUCLEOTIDE, AND APPARATUS AND ARRAYS CONTAINING A PLURALITY OF SAME

Group Art Unit: 1634
Former Group Art Unit: 1809
Examiner: Ardin H. Marschel, Ph.D

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Honorable Commissioner of Patents and Trademarks
The United States Patent and Trademark Office
Washington, D.C. 20231

SUPPLEMENTAL DECLARATION OF DR. DEAN L. ENGELHARDT IN SUPPORT OF THE NOVELTY OF INVENTION AND CLAIMED SUBJECT MATTER

I, Dean L. Engelhardt, Ph.D, hereby declare as follows:

1. I am currently employed by Enzo Biochem, Inc., 527 Madison Avenue, New York, New York 10022 as Senior Vice President, having held that position since 1988. Prior to my employment at Enzo Biochem, Inc., I was Associate Professor of Microbiology at Columbia University College of Physicians and Surgeons, New York City, having earlier obtained my doctorate from Rockefeller University in New York City. A copy of my curriculum vitae (C.V.) was attached as Exhibit A to my earlier Declaration to the Patent Office submitted on July 21, 1998. The title of that paper was "Declaration of Dr. Dean L. Engelhardt in Support of Possession of Claimed Subject Matter And Novelty of Invention."

2. I continue to serve in my position as Senior Vice President of Enzo Biochem, Inc., having also served as its Director of Research in which capacity I have overseen scientific research activities for the company and its subsidiaries. I also continue to oversee various research projects, including those currently under my direction relating to research and human clinical trials in connection with Enzo's IND application submitted last year to the Food and Drug Administration (FDA). The IND applications concerns the use of genetic antisense nucleic acid medicine for HIV-1.. Among my other responsibilities at Enzo Biochem, Inc. have been the development of new nucleic acid technology and hybridization formats, including new diagnostic and therapeutic approaches and agents based upon nucleic acid technology.

3. I am familiar with the contents of this application and portions of its prosecution history as noted in my earlier Declaration. I understand that claims 48-100 and 102-182 represent the presently pending claims in the application, and that amendments to claims 48, 77, 80, 100 and 102 were effected in a Supplemental Amendment to Applicants July 21, 1998 Amendment Under §1.115 that was filed on August 17, 1998. Claims 48-100 and 102-182 are directed variously to a composition of matter, a system, an apparatus and an array. Among the claims are a composition of matter represented by independent claims 48 and 77, a transparent non-porous or translucent non-porous system represented by independent claim 102, an apparatus set forth in independent claims 100 and 132, and an array that is recited in independent claim 141. As set forth below, each of the aforementioned independent claims defines the soluble signal as a quantifiable element that is generated or generatable from a chemical label or labels which comprise a signalling moiety or moieties.

> 48. A composition of matter comprising:
> a transparent non-porous or translucent non-porous system containing a fluid or solution, which system comprises:
> (i) a solid support; and
> (ii) a double-stranded oligonucleotide or polynucleotide which is directly or indirectly fixed or immobilized to said solid support wherein one of the strands produces a quantifiable soluble signal generated or generatable from a chemical label or labels which comprise a signalling moiety or moieties.

77. A composition of matter comprising:

a transparent non-porous or translucent non-porous system containing a fluid or solution, which system comprises:
a double-stranded oligonucleotide or polynucleotide which is directly or indirectly fixed or immobilized to said system wherein one of the strands produces a quantifiable soluble signal generated or generatable from a chemical label or labels which comprise a signalling moiety or moieties.

100. An apparatus comprising:

1) one or more solution containing means, each comprising a transparent non-porous or translucent non-porous device;

2) means for forming a fixed or immobilized double-stranded oligonucleotide or polynucleotide hybrid to a solid support in said device, said hybrid comprising a chemical label or labels attached to one strand of said hybrid, said label or labels comprising a signalling moiety or moieties which are capable of generating a quantifiable soluble signal; and

3) means for producing a quantifiable soluble signal generatable or generated from said chemical label or labels which comprise said signalling moiety or moieties.

102. A transparent non-porous or translucent non-porous system containing a fluid or solution, which system comprises:

(ii) an oligonucleotide or polynucleotide hybridized or hybridizable to an oligo- polynucleotide sequence, said oligonucleotide or polynucleotide in double-stranded form producing a quantifiable or detectable soluble signal generated or generatable from a chemical label or labels which comprise a signalling moiety or moieties; and

(ii) a solid support having directly or indirectly fixed or immobilized thereto said oligo- or polynucleotide sequence or said oligonucleotide or polynucleotide (i).

132. An apparatus comprising:

1) means for retaining or containing a fluid or solution;

2) one or more transparent non-porous or translucent non-porous devices, each comprising a solid support;

3) means for forming a fixed or immobilized oligonucleotide or polynucleotide hybrid to said solid support, said hybrid comprising a chemical label or labels attached to said hybrid, said label or labels further comprising a signalling moiety or moieties capable of generating a soluble signal;

4) means for quantifying or detecting a soluble signal generatable or generated from said chemical label or labels comprising said signalling moiety or moieties; and

5) fluid or solution.

4. As noted in my earlier Declaration, I read the two Office Action dated January 21, 1998 and October 2, 1995 that were issued in connection with this application. The Examiner's remarks in three anticipation rejections covering four

prior art documents (Stuart et al., U.S. Patent No. 4,732,847; Langer-Safer et al. [PNAS 79:4381-4385 (1982); Manuelidis et al. [Journal of Cell Biology 95:619-625 (1982)]; and Ward, U.S. Patent No. 4,711,955) were given as follows in the January 21, 1998 Office Action:

> Applicants argue the below art rejections in that the practice of in-situ hybridization is a very specialized type of methodology and different from the soluble signal generation practice as instantly claimed. In response applicants are reminded that compositions, apparati, and systems are claimed and not methods. Therefore, if a reference meets the composition, apparatus, or system limitations, it anticipates the instant invention even if a number of uses can be practiced for the claimed invention. In other words patentable weight is not given to use limitations if they do not limit the actual composition etc. limitations.
>
> Applicants argue that Stuart et al. is concerned with in situ hybridization and not applicable. This is non-persuasive as already discussed above. Applicants then argue that the instant invention requires chemically labeled nucleic acid for one of the strands. This is also non-persuasive because there is therein no limitation regarding whether a label may or may not also interact with the second strand in a hybrid formed during a hybridization assay. This rejection is reiterated and necessitated by amendment due to the newly added claims.
>
> The listed claims are anticipated either by Langer-Safer et al. or Manuelidis et al. in the same manner as the above rejection based on Stuart et al. because both references also discussed the performance of in situ hybridization of chromosome spreads on microscope slides etc. as summarized above. This rejection is reiterated and necessitated by amendment due to the newly added claims.
>
> Ward et al. disclose via the "GENERAL PROTOCOL" and with connected discussion elsewhere at the bottom of columns 19 and 20 in situ hybridization where immobilized double-stranded nucleic acid is shown visualized with a biotinylated probe bound to avidin-peroxidase. This reads on the listed claims as the peroxidase is capable of generating a soluble signal as discussed above and therefore still reads on the instant invention even though Ward et al. discloses insoluble signal generation. This rejection is reiterated and necessitated by amendment due to the newly added claims.

C. As also noted in my previous Declaration, I understand that in the October 2, 1995 Office Action, anticipation rejections based upon the four cited documents, Stuart et al., Langer-Safer et al., Manuelidis et al. and Ward et al. were also made. The Examiner's position on the anticipation issues was stated as follows in the October 2, 1995 Office Action:

Stuart et al. disclose the formation of DNA-RNA hybrids by in situ hybridization performed on microscope slides wherein the hybrids are detected by antibody binding to said hybrids starting in column 4, line 1, and proceeding through the section entitled 'EXPERIMENTAL". Several types of antibody label types are given in Stuart et al. in column 4, lines 37-55, inclusive of enzymes such as horseradish peroxidase. These disclosures read on the above listed instant claims. It is additionally noted that microscope slides are non-porous and transparent and are disclosed as being viewed by light microscopy as summarized in column 6, lines 17-26, thus also inclusive of a "system" as instantly claimed. Such a light microscope system includes non-porous transparent elements which are interpretable as reading on these limitations of the system of the instant claims. It is also noted that Stuart et al. reads on the above listed instant claims after removal of the NEW MATTER discussed above. The above listed claims contain the limitation directed to "a signalling moiety which is capable of generating a soluble signal". Enzymes such as horseradish peroxidase inherently are "capable of generating a soluble signal" as is well known in the peroxidase labeled antibody art. It is acknowledged that Stuart et al. does not disclose the actual generation of a soluble signal. This lack of actual soluble signal generation is, however, not deemed to prevent this rejection because the claims are composition claims citing only the "capability" of generating such a soluble signal. The inherency of this "capability" has been discussed above.

The listed claims are anticipated either by Langer-Safer et al. or Manuelidis et al. in the same manner as the above rejection based on Stuart et al. because both references also discussed the performance of in situ hybridization of chromosome spreads on microscope slides etc. as summarized above. It is noted that Langer-Safer et al. has been listed on a PTO Form 1449, filed by applicants on 6/2/94, as Langer et al. (1982) as given in PNAS, volume 79, pages 4381-4385. The correct citation is to Langer-Safer et al. and has also been corrected on the PTO Form 1449.

Ward et al. disclose via the "GENERAL PROTOCOL" and with connection discussion elsewhere at the bottom of columns 19 and 20 in situ hybridization where immobilized double-stranded nucleic acid is shown visualized with a biotinylated probe bound to avidin-peroxidase. This reads on the listed claims as does the above disclosures directed to in situ hybridization but also covering biotin-avidin mediated embodiments.

5. I am making this Declaration in support of the novelty of the invention defined by the pending claims, particularly reflected by the amendments submitted on July 21, 1998.

6. Each of the pending claims is directed to subject matter in which a quantifiable soluble signal is generated or generatable from a signaling moiety or

moieties of a chemically labeled double-stranded oligonucleotide or polynucleotide which is directly or indirectly fixed or immobilized to a solid support which is in a transparent non-porous or translucent non-porous system (claims 48-76 and 86, 109, 133, 135, 138 and 140, and 102-108, 110-131, 134, 137, 139 and 142), or is directly or indirectly fixed or immobilized directly to such system (claims 77-85, 87-99, 133, 136 and 141). The apparatus of claims 100 and 132 also include the element of a means for producing a quantifiable soluble signal generated or generatable from such chemical label or labels comprising a signaling moiety or moieties. It is my opinion and conclusion that none of the documents cited against the pending claims disclose the instantly claimed element of a quantifiable soluble signal generated or generatable from a chemically labeled oligonucleotide or polynucleotide.

7. The remarks that follow below are presented as background to the *in situ* hybridization prior art documents cited against this invention.

A. As an investigative tool, *in situ* hybridization (ISH) has been around for almost three decades with three groups, including Buongiorno-Nardelli and Amaldi (1969), Gall and Pardue (1969) and John et al. (1969), having been largely credited for their early work in this area. Early work in ISH relied on autoradiography and that continued until the advent of non-radioactive nucleic acid labeling and detection in the 1980s. A copy of each of three 1969 papers from these groups are attached as Exhibits 1, 2 and 3, respectively. ISH represents a significant departure from other hybridization detection formats, including conventional filter and solution hybridization.

B. Several nucleic acid textbooks provide exquisite accounts of ISH including three now described:

(i) A thoroughly informative description of *in situ hybridization* is provided by Piper and Unger:

(a) who explain that *in situ* hybridization

> . . . *involves taking morphologically intact tissues, cells, or chromosomes through the hybridization process to demonstrate not only the presence of a particularl piece of genetic information, but also its* ***specific location*** *within the tissue, cell, or chromosome*. In situ hybridization techniques represent the best compromise between making the nucleic acid target available for hybridization and ***maintaining the morphologic integrity*** *of the starting material*. The target nucleic acids are found intimately mixed with the proteins, other nucleic acids, and membranes that form the basis of the familiar staining patterns with "routine" stains such as H&E, Papanicolaou, or Giemsa banding. The goal is to make the target nucleic acid available for hybridization while **maintaining a recognizable "staining environment"** so that the tissue, cell, or chromosome can be **identified by the landmarks of routine staining**.

[Nucleic Acid Probes: A Primer for Pathologists, Margaret A. Piper and Elizabeth R. Unger, ASCP Press, American Society of Clinical Pathologists, Chicago, 1989, Chapter 2, "Nucleic acid hybridization analyses and other nucleic acid assays," pages 61-66, emphasis added; copy attached as Exhibit 4]

(b) Piper and Unger explain with *in situ* hybridization, the methods for affixation of the sample starting material to the viewing slide is critical:

> Sample preparation involves some method of affixing the starting material to a microscope slide, *because* ***light microscopic examination is required*** *to evaluate the assay*. The slide becomes the solid support that carries to the cells, tissue, or chromosomes through all of the following steps of the hybridization assay. ***This step is not trivial*** *because the conditions of the hybridization assay can be quite harsh and loss of sample through detachment during the assay is a major concern*. Adherence is improved by coating the glass with substances such as gelatin, polylysine, aminopropylsilane, or Elmer's Glue.
>
> . . .
>
> The sample type and method of preparation greatly influence the details of the technique. Fresh or frozen material will behave much differently from fixed material, particularly if a cross-linking fixative such as formaldeyde or glutaraldehyde is used. *Fixatives were developed to* ***preserve morphology*** *through routine staining procedures, and thus will add to the* ***morphologic integrity*** *of the finished hybridization product*. At the same time, fixatives will decrease the availability of the nucleic acid that is to undergo hybridization.
>
> [Piper & Unger, *ibid*., pages 62-63, emphasis added, Exhibit 4]

(c) Because morphology, morphologic integrity and landmarks are vital to *in situ* hybridization, *supra*, labeling and sample treatment

are crucial to evaluating and interpreting *in situ* results. In the case of labels for *in situ* hybridization, Piper and Unger explain:

> Detection of the hybridization signal depends on the method used to label the probe. Various radioactive labels may be used; all are detected with silver emulsion autoradiography. Exposure times range from overnight to several months. With low-energy radioisotopes such as tritium, there is ***minimal scattering*** *of the silver grains from the site of probe localization* but the required exposure times are the longest. High-energy isotopes such as ^{125}I give very poor localization of signal but require very short development times. In practice, ^{35}S is a useful compromise between signal localization and development time.
>
> With nonradioactive labels the final detection is analogous to methods used in immunochemistry: either the fluorescent tags or histochemical enzymes may be utilized *to* ***localize*** *the position of the reporter molecule on the probe. There is essentially* ***no scatter of signal*** *with either method.* The results of fluorescence may be viewed immediately, while enzyme-derived colorimetric products require 10 minutes to overnight for development. Colorimetric or fluorescent analyses are generally much faster than autoradiography, but many investigators believe there is a significant loss of sensitivity. Others feel that with careful optimization of the assay conditions, nonradioactive methods can achieve sensitivities very close to those obtained with radioactive isotopes.
>
> [Piper & Unger, *ibid.*, page 65, emphasis added, Exhibit 4]

(d) Regarding evaluation and interpretation of *in situ* results, Piper and Unger further explain:

> After counterstaining *to bring out the* ***morphology*** *and maximize contrast between the signal and surroundings, the final product is evaluated by light or fluorescence microscopy. Through visual inspection the presence of signal is determined qualitatively, and it is* ***localized to a particular cell or chromosome***. Autoradiographic detection can be made semiquantitative by counting the number of developed silver grains per area. Semiquantitative colorimetry can be accomplished with computer-assisted image analysis and morphometry. Attempts at quantitation can be quite complicated, and most investigators use *in situ* hybridization at the qualitative level.
>
> *In situ hybridization occurs and is* ***interpreted in a morphologic context***. Unlike hybridization assays based on extraction techniques, the sensitivity of the in situ assay is influenced not only by the number of copies of the target sequence but also by its distribution in a sample, *while in situ methods are most sensitive when target sequences are nonuniformly distributed.* When only one or two cells in a relatively large sample contain the target sequences, extraction

> methods dilute those positive sequences, while in situ hybridization assays preserve the natural concentration and easily detect the few positive cells in a negative background. This is the only form of hybridization that combines the power of morphologic analysis with a sophisticated genetic analysis and *permits **definitive localization of** genetic information*.
>
> [Piper & Unger, *ibid.*, pages 65-66, emphasis added, Exhibit 4]

(e) *In situ* hybridization is generally unsuitable for handling large numbers of samples. In closing the section on *in situ* hybridization, Piper and Unger note:

> *The in situ hybridization assay can be quite **tedious** and each sample must be **treated individually**. This makes the handling of multiple samples very difficult and is one of the biggest drawbacks of the assay. Because **morphologic integrity** is crucial to the assay, investigators familiar with extraction-based techniques often find in situ hybridization to be a kind of "black magic" that is subject to many more variables influencing both signal and background*. With experience in working with tissues, cells, and chromosomes, these difficulties usually can be resolved; however, the assay does have an extra level of complication compared with extraction-based techniques.
>
> [Piper & Unger, *ibid.*, page 66, emphasis added, Exhibit 4]

(ii) Larry Kricka provides the following description:

> *In situ* hybridization has become an important tool in cellular and molecular biology. *Conventional filter and solution hybridization methodologies rely on isolation of nucleic acids from a population of cells, thereby averaging the information from each individual cell with the total contribution of all cells. However, in situ hybridization provides a tool for studying the **molecular information of individual cells within a tissue or cell population**. This also allows the investigator to correlate the molecular information with the morphological markers in individual cells*. *In situ* hybridization has been used extensively to reveal information regarding expression of particular genes (Lawrence and Singer, 1986), the presence of infectious agents (Hasse, *et al.* 1984), or the location of particular genes in chromosomes (Lawrence *et al.*, 1990).
>
> Both radioactive and nonradioactive probes have been utilized for *in situ* hybridizations. (Singer *et al.*, 1987). Biotinylated probes are the most widely used nonradioactive system. In brief, a biotin-labeled probe is hybridized to target DNA or RNA in cells or tissues *in situ* on a microscope slide. A signaling group (alkaline phosphatase) covalently attached to streptavidin is then bound to the biotinylated probe. The hybridized probe is detected by incubating the samples with dye substrates for alkaline phosphatase, NBT and BCIP.

> Formation of a purple signal indicates the location of the hybridized probe.

[Nonisotopic DNA Probe Techniques, Larry J. Kricka, editor, Academic Press, Inc., San Diego & New York, 1992, Chapter 5, pages 155-156, emphasis added, copy attached as Exhibit 5]

(iii) Ten years after the initial patent application was filed, Mark Manak gives the following introduction to *in situ* analysis:

> *In situ hybridization methods allow the* ***microscopic examination of specific DNA or RNA sequences in cells preserving the relative location of these sequences in the various cell types within a tissue and even within specific structures or their location on chromosomes.*** *This technique offers many important advantages which are not available when using nucleic acids extracted from cells which become mixed and diluted with the nucleic acids of adjacent cells*. Individual cells in a complex tissue can be examined for the expression of specific mRNAs and the distribution of mRNA expression within them. It is possible to analyze many types of tissues simultaneously without having to purify RNA from them. The amount of material required for analysis is signficantly lower than needed for filter based studies, permitting meaningful examination of even small biopsy samples.

[DNA Probes: Background•Applications•Procedures, Second Edition, George H. Keller and Mark M. Manak, Stockton Press, 1993, New York, Section 8, "In Situ Analysis," M. Manak, page 299; emphasis added, copy attached as Exhibit 6]

Later on the same page, the following description of *in situ* hybridization is offered:

> The *in situ* hybridization method is well suited for cytology and histology laboratories and is finding applications in other settings as well. The microscopic examination of the specimen *allows detection of small amounts of hybridization signal in a* ***well defined area***. . .
>
> [DNA Probes, page 299, emphasis added, Exhibit 6]

C. A number of textbooks devoted entirely to *in situ* hybridization have also been published, including two contributed by David G. Wilkinson.

(i) In the first, Dr. Wilkinson provides the following excellent introduction:

> *The principle behind in situ hybridization is the specific annealing of a labelled nucleic acid probe to complementary sequences in fixed tissue*, followed by visualization of the location of the probe. This technique can be used to locate DNA sequences on chromosomes, or to detect RNA or viral DNA. An important asset is the speed with which specific probes for *in situ* hybridization, all involving a series of procedures:
> - preparation of probe
> - preparation of tissue
> - hybridization of probe to tissue and washing
> - visualization of probe
>
> *A critical aspect of these procedures is that the target nucleic acid is* ***retained in situ***, is not degraded by nucleases, and is accessible for hybridization to the probe. *Unlike the hybridization of nucleic acids in solution, the target nucleic acid is cross-linked and embedded in a complex matrix* that hinders access of the probe and decreases the stability of the hybrids. . .

[*In Situ* Hybridization: A Practical Approach, edited by D. G. Wilkinson, IRL Press at Oxford University Press, Oxford and New York, 1992, Chapter 1, "The theory and practice of *in situ* hybridization," David G. Wilkinson, page 1; emphasis added, copy attached as Exhibit 7].

(ii) In the second account, also edited by Dr. Wilkinson, the following introduction to ISH is given:

> **Introduction**
> *In situ* hybridization (ISH) may be defined as the *direct detection of nucleic acid in intact cellular material*. Nucleic acids may be exogenous or endogenous, nuclear or cytoplasmic, DNA or RNA. A variety of cell and tissue samples can be studied using ISH, from individual chromosomes in metaphase spreads to archival paraffin embedded biopsy material. Using appropriately labelled probes, the presence or absence of normal and abnormal nucleic acids can not only be detected but can also be *correlated with* ***cell and tissue morphology***. . .

[*In Situ* Hybridisation: Application to Developmental Biology and Medicine, edited by N. Harris and D. G. Wilkinson, Cambridge University Press, Cambridge and New York, 1990, "Non-isotopic *in situ* hybridisation in human pathology," Herrington et al., page 241; emphasis added, copy attached as Exhibit 8]

D. Several scientific and technical dictionaries provide definitions that comport with the above descriptions and definitions for *in situ* hybridization:

(i) Stenesh defines the term thusly:

> **in situ hybridization** A technique for ***localizing specific DNA segments within intact chromosomes***; involves incubating chromosomes, devoid of RNA and protein, with tritium-labeled nucleic acid and visualizing the hybridized segments by radioautography.

[Dictionary of Biochemistry and Molecular Biology, Second Edition, J. Stenesh, John Wiley & Sons, New York, 1989, page 244; emphasis added, copy attached as Exhibit 9].

(ii) Another definition is given by Rieger et al.:

> **in situ nucleic acid hybridization** (Gall and Pardue 1969; John et al. 1969) - the annealing (hybridization) of radioactive single-stranded DNA or RNA probes to denatured cellular DNA on microscopic slides and their detection by autoradiography. In situ n.a.h. (= cytological h.) *permits the **localization** of DNA sequences (genes) complementary to the probes in **morphologically intact chromosomes***. Biotin-labeled probes may also be used for i.s.n.a.h. (higher resolution of hybridization sites and lower background interference than with conventional hybridization.

[Glossary of Genetics, Classical and Molecular, Fifth Edition, R. Rieger et al., Springer-Verlag, Berlin and New York, 1991, page 271; emphasis added, copy attached as Exhibit 10].

E. Dr. David C. Ward of Yale University, a pioneer in the field of non-radioactive labeling and detection of nucleic acids, and a recently elected member of the National Academy of Sciences, has also provided countless descriptions of *in situ* hybridization, including the following:

(i) from his 1982 PNAS paper:

> The method of *in situ* hybridization has been a powerful tool for the ***localization of specific sequences linearly arranged in a chromosome*** (1). This approach has been modified for the ***detection of RNA transcripts within individual cells***. . .

[Singer, R. H. and David C. Ward, "Actin gene expression visualized in chicken muscle tissue culture by using *in situ* hybridization with a biotinated nucleotide analog,""Proc. Natl. Acad. Sci. (USA) 79:7331-7335 (December 1982), page 7331; emphasis added; copy attached as Exhibit 11]

(ii) his 1987 Acta Histochem. Cytochem. paper:

> *In situ* hybridization yields information that is not readily obtained with other nucleic acid technologies. In particular, complex cell populations may be evaluated for the presence of specific nucleic acid sequences and these molecules ***detected in selected individual cells to which they may be confined***. Thus, the sensitivity of *in situ* hybridization in mixed cell populations can be considerably greater than that achieved with filter blotting assays. . . The ability to describe the relative expression and/or ***localization*** of two distinct nucleic acid sequences is important, for instance for the study of the temporal pattern of gene expression during developmental transitions or viral infections, and for investigating gene arrangements in chromosomes or interphase nuclei.

[Singer et al., "Double Labelling In Situ Hybridization Using Non-Isotopic And Isotopic Detection," Acta Histochem. Cytochem. 20:589-599 (1987); emphasis added; copy attached as Exhibit 12]

8. As recognized by the Examiner in the January 21, 1998 and October 2, 1995 Office Actions, each of the four documents (Stuart et al., Langer-Safer et al., Manuelidis et al. and Ward et al.) which were cited against the claims in this application are disclosures directed to *in situ* hybridization. As such, each document is limited to *in situ* hybridization which can only be practiced in the context of clear boundaries and well-defined morphology against which a localized signal - and not a soluble signal let alone a quantifiable soluble signal - must be produced and interpreted. The quantifiable soluble signal which is a material element in the pending claims of this application is in no way localized nor is morphology either required, maintained or viewed. Indeed, with the generation of a quantifiable soluble signal, a dispersed or scattered signal in solution is obtained without any regard to any limitation or requirement for morphologic integrity. Based upon my many years of experience in the field, my education, and professional and academic background, it is my conclusion and position that with their preoccupation on morphologic integrity (landmarks, boundaries and the like) and a localized signal (and not a quantifiable soluble signal), the four prior art *in situ* hybridization documents actually teach away from the invention set forth in the present claims. As a material element in the present invention, the quantifiable soluble signal requires neither localization nor morphologic integrity of the prior art *in situ* hybridization disclosures.

9. Turning to Stuart et al., the first of the four cited documents, it is my

opinion and conclusion that this patent lacks at least two material elements defined by the present claims.

A. **First**, Stuart et al. is wholly and only concerned with *in situ* hybridization and thus, requires morphologic integrity and a localized signal - not a quantifiable signal as defined by the present claims of this application. More particularly, Stuart et al. disclose *in situ* hybridization to form a hybrid DNA-RNA duplex between a nucleic acid sequence (DNA or RNA) fixed to a solid support and another type of nucleic acid (RNA or DNA) having a predetermined sequence to bind to a homologous fixed sequence. Having obtained such a hybrid duplex through *in situ* hybridization, Stuart then uses labeled hybrid duplex monoclonal antibodies (or labeled antibodies which bind to the "anticomplex") to detect the presence of the fixed specific nucleic acid sequence. See Stuart et al., column 1, lines 65-68; column 3, lines 41-42; column 4, lines 24-35; column 5, lines 2-4 (in the EXPERIMENTAL section); column 5, lines 60-68 (in the subsection "In Situ Hybridization"); and column 7, lines 14-16.

B. **Second**, unlike the instant invention which calls for and requires a chemical label or labels on one of the nucleic acid strands, Stuart et al. do not disclose or even suggest any such modification for any nucleic acid - and that is because Stuart's is an immunoassay. Stuart et al. rely exclusively on labeled monoclonal antibodies (or antibodies in the case of the "anticomplex") which are specific for the hybrid DNA-RNA complex. Stuart et al. employ such labeled monoclonal antibodies (or antibodies) for the sole purpose of detecting *immunologically* the hybrid DNA-RNA complex. This is clearly seen at column 4, lines 24-55 in Stuart's patent where it is disclosed:

> The identification of the presence of the hybrids may now be achieved by employing monoclonal antibodies specific for the hybrid complex. Detection can be achieved *by labeling either the monoclonal antibody specific for the hybrid DNA-RNA complex,* hereinafter referred to as 'anticomplex" or *by employing labeled antibodies which bind to the anticomplex.* For example, where the monoclonal antibody is derived from a mouse, antibodies to mouse antibodies e.g. rabbit anti(-mouse IgG), could be labeled so as to bind to any anticomplex bound to the complex bound to the solid support. A wide variety of labels have been used in other environments which would be applicable here. One of the more common labels is

> radionuclides, which can be used with autoradiography to visualize the areas of binding. Another label is a fluorescer e.g. fluorescein, merocyanine, rhodamine, etc., which by irradiation with light of excitation, the presence of fluorescence can be monitored. Alternatively, *an enzyme can be used which results in a product which can be detected and **localized in the area** of the enzyme*. A large number of dyes or metals capable of reduction can be employed to provide detection. Common enzymes include horseradish peroxidase, glucose oxidase, β-galactosidase, or the like. The particular label or manner in which the detectable signal is observed is not critical to this invention. Evidently, by employing antibodies to this anticomplex, the number of labels associated with a particular binding of the anticomplex to the complex can be greatly amplified.
>
> [underline, italic & bold added]

Later in their example, Stuart et al. disclose the identification of hybrids by secondary immunofluorescence. More specifically, they disclose that "[g]oat antirabbit and goat anti-mouse Ig antisera conjugated with fluorescein isothiocyanate were purchased from Antibodies, Inc." [column 6, lines 19-21]. Furthermore, "[w]hen the RNA normally present in the polytene preparation was not predigested with Rnase A, multiple fluorescent bands were observed after hybridization. This evidences that the antibodies were not reactive with chromosomal DNA but were able to bind to RNA-DNA duplexes" [column 6, lines 31-36]. See also claim 5 in Stuart et al. (. . . determining the presence of monoclonal antibody bound to said solid support by means of a label providing a detectable signal, which label is bound directly or indirectly to said monoclonal antibody), and claim 14 (kit for determining the presence in situ of a hybrid DNA-RNA complex . . . said secondary antibodies having a label capable of providing a detectable signal). Thus, Stuart et al. only contemplate labeling the monoclonal antibodies (or antibodies in the case of the "anticomplex"). Such labelling is in contrast to the instant invention in which one of the oligonucleotide or polynucleotide strands bears a chemical label or labels.

10. As in the case of the previous anticipation rejection based on Stuart et al., Langer-Safer et al. and Manuelidis et al. are likewise confined to *in situ* hybridization. As such, both articles teach away from the instant invention because *in situ* hybridization requires morphologic integrity and localized signal production - and not a quantifiable soluble signal as embodied materially in the present claim elements. This distinction between *in situ* hybridization, immunoprecipitates and insoluble signal on the one hand, and the instantly recited

quantifiable soluble signal on the other, is a significant distinction. When performing *in situ* hybridization, the technician or researcher is looking under the microscope and observing form or morphology as well as signalling events within the context of any such form or morphology. Such a person only observes and amasses information within the context of clearly defined boundaries and visible shapes. The cells or the contents of cells under examination must have such boundaries and shapes in order to carry out detection in *in situ* hybridization. Separation and analytical techniques are largely based on precipitates and defined boundaries. With soluble signals, however, the approach is entirely antithetical to the purposes of and the information being sought through light microscopic examination. With a quantifiable soluble signals, however, there are no clearly defined boundaries, shapes and morphology with which the technician or researcher must contend. In fact, the technician or research is concerned with the total amount of target nuclec acid analyte in the sample or specimen - and not with its location or distribution in the cells or tissues. Thus, *in situ* hybridization as disclosed in the Langer-Safer and Manuelidis et al. documents teaches away from the instant invention. By directing and focusing attention to morphology and localized signaling, both of which are required in *in situ* hybridization, Langer-Safter et al. and Manuelidis et al. actually discourage any resort to a quantifiable soluble signal. Indeed, for the skilled artisan to go from precipitates and insoluble signals to a quantifiable soluble signal, all prior conceptions and experiences relating to detection (localized signals) and observation (morphologic integrity) would have to be abandoned altogether.

11. As in the case of the other three documents cited in the two office actions, Ward et al. is also wholly concerned with immunoprecipates, precipitated signals, insoluble colored precipitates and the like.

A. For example, in column 21, first paragraph (lines 4-24), Ward et al. disclose:

> Modified nucleotides may be used in a method of gene mapping by in situ hybridization which circumvents the use of radioisotopes. This procedure takes advantage of a thymidine analogue containing biotin that can be incorporated enzymatically into DNA probes by nick translation. After hybridization in situ the biotin molecules serve as

> antigens for affinity purified rabbit anti-biotin antibodies. Immunofluorescent antibody sandwiches made with fluorescein-labeled goat anti-rabbit IgG allow for rapid and specific cytogenetic localization of cloned gene sequences as green-yellow bands. This method offers four major advantages over conventional autoradiographic methods of in situ gene localization; less background noise, an increase in resolving power between bands; a decrease in the time required to determine the size of probe hybridization; and chemically stable hybridization probes. This method has been applied successfully to the localization of reiterated and unique DNA sequences in the polytene chromosome of *Drosophila melanogaster* and satellite DNA on mouse metaphase chromosomes.

B. Regarding Ward's "GENERAL PROTOCOL," it is quite clear that the patentees are working with the enzyme peroxidase in what are clearly insoluble precipitates for light microscope visualization. In column 24, lines 33-62, Ward et al. disclose:

> An alternative to the fluorescence method for visualizing hybridized probes is to direct enzymes such as peroxidase, alkaline phosphatase of[or] β-galactosidase to the hybridization site *where enzymatic conversion of soluble substrates to* ***insoluble colored precipitates*** *permits light microscope examination*. The important advantage of this technique is that the histochemical methods are 10 to 100-fold more sensitive than fluorescence detection. In addition, the ***colored precipitates*** do not bleach with extensive light exposure thus avoiding one of the general disadvantages of fluorescent light microscopy. These enzymes can be coupled to the final antibody instead of using bifunctional reagents such as glutaraldehyde or in the case of peroxidase via oxidation of the peroxidase carbohydrate moieties to aldehydes and coupling of these residues with e-amino groups of the desired protein. For the streptavidin-biotinized carrier protein method, an enzyme with biotinyl groups coupled to it could replace a fluorescently-biotinized carrier system. Alternatively, the enzyme could be coupled via biotin to the last layer of streptavidin with amplification of streptavidin sites being built up in preceding layers using biotinized BSA or thyroglobulin. We will begin developing the necessary histochemical reagents and the appropriate *combinations substrate/insoluble product combinations* for visualizing in situ hybridizations without background problems in the near future. The histochemical approaches to signal amplification should therefore be ready for trial in the summer of 1981. [emphasis added]

It is my opinion and conclusion, therefore, that the focus of Ward et al. is on insoluble colored precipitates and direct light microscopic visualization which elements are diametrically opposed to and actually teach away from the present invention and the notion of a quantifiable soluble signal.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that any such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Sept 21, 1998
Date

Dean L. Engelhardt

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